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Micrometastatic Tumour Cells in Bone Marrow of Patients with Gastric Cancer: Methodological Aspects of Detection and Prognostic Significance

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Monoclonal antibodies (Mab) are potent probes to identify individual tumour cells or small tumour cell clusters in bone marrow. In the present study, various antibodies directed against either cell surface or intracytoplasmic antigens of epithelial cells were assessed for their ability to detect such cells in bone marrow of patients with breast, colorectal and gastric cancer. According to the presented data, monoclonal antibodies against intracellular cytokeratin (CK) components are superior in terms of specificity and sensitivity to antibodies reacting with epitopes of the cell membrane. Using a monoclonal antibody against the cytokeratin polypeptide 18 in connection with the alkaline phosphatase anti-alkaline phosphatase detection system (APAAP), we could detect tumour cells in bone marrow of 34 out of 97 patients with gastric cancer examined at the time of primary surgery. The incidence of positive findings was correlated to established risk factors, such as histological classification and locoregional lymph node involvement. Clincal follow-up studies on 38 patients demonstrated a significantly increased relapse rate in patients presenting with CK-positive cells in their bone marrow at the time of primary surgery. Thus the described technique may help to identify patients with gastric cancer carrying a high risk of early relapse.
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INTRODUCTION

THE TREATMENT of the most common types of malignant tumours has hardly improved during the last decade. This overall negative balance is mainly due to the systemic dissemination of tumour cells occurring often prior to the diagnosis or resection of the primary tumour. Thus, the detection and elimination of micrometastases in patients with small resectable tumours poses a major challenge to cancer research.

Monoclonal antibodies (Mab), directed against epithelial differentiation antigens are able to identify individual epithelial tumour cells or small carcinoma cell clusters in mesenchymal organs, which are undetectable by conventional diagnostic methods. The bone marrow presents as an easily accessible mesenchymal organ and tissue compartment from which epithelial cells appear to be rigorously excluded in non-malignant conditions. It is evident that an immunocytochemical assay depends on the specificity of the antibody and on the sensitivity of the applied detection system. The antibody used should be

directed against an antigen preferentially expressed on all tumour cells and should not react with haematopoietic or other autochthonous cells of the bone marrow. These conditions are met by monoclonal antibodies directed against epitopes on cytokeratin, which as an abundant intracellular protein antigen appears to be a target for cell identification superior than cellular membrane antigens. For the present study, selected antibodies were applied to detect micrometastatic tumour cells in bone marrow of 97 patients with gastric carcinoma. In this common type of cancer with its rather unfavourable prognosis, systemic dissemination of tumour cells could be frequently detected at the time of primary surgery.

PATIENTS AND METHODS

Patients

139 patients with histologically proven primary carcinoma (97 gastric cancer, 25 breast cancer and 17 colorectal cancer) were analysed. After an extensive diagnostic programme, including chest X-ray, ultrasound and computed tomography (CT) of the abdomen as well as bone scan, all patients were staged according to the primary tumour/regional lymph-nodes/metastases (TNM) classification. One marrow sample was aspirated from the posterior iliac crest of each patient at the time of primary surgery. Bone marrow aspirates were also obtained from 75 patients without cancer. Informed consent was obtained from all participating patients.

Immunocytochemistry

A mean volume of 5.5 ml bone marrow per aspiration was obtained yielding an average of 1.0×10^7 nucleated cells. The mononuclear cells were isolated by density centrifiguation through Ficoll/Hypaque. The interface cells were washed by centrifugation and resuspended in phosphate-buffered saline (PBS). Finally the cells were centrifuged on glass slides with a cytocentrifuge. The average number of cells per slide was 3×10^4. After acetone fixation (10 min) and air drying for 12–24 h the slides were either stained immediately or stored at −70°C. Routinely, five slides comprising about 1.5×10^5 nucleated cells were examined for each patient, an additional slide served as control for staining with immunoglobulin (Ig) isotype.

The following Mab were tested for immunocytochemistry application: Mab HMFG1 (IgG) and HMFG2 (IgG) (kindly provided by Dr J. Taylor-Papadimitriou, London), directed against glycoprotein epitopes on human milk fat globules (HMFG), are reactive with cell surface antigens on epithelial cells [1, 2]. Mab E 29 (IgG) (Dako, Hamburg) detects a related epithelial membrane antigen (EMA) that is widely distributed in epithelial tissues and carcinomas [3]. Mab M 77 (IgG 2b) and M79 (IgG2a) recognise different epitopes of the 17-1A antigen (37-KD glycoprotein) on the cell membrane of epithelial cells. These antibodies were used in a 1:1 mixture [4]. Mab Lu5, CK8, CK2 and KS 19.1 are directed against components of the intermediate filaments of cytokeratin type. Cytokeratin polypeptides are essential constituents of the cytoskeleton of both normal and malignant cells [5]. Mab Lu5 (IgG1) (Boehringer Mannheim) reacts with an epitope which is common to all cytokeratins with a cytokeratin associated protein [6]. Mab CK8 (Boehringer Mannheim) recognizes cytokeratin component 8 [7], Mab CK2 (IgG1) (Boehringer Mannheim) detects cytokeratin component 18 [8, 9] and Mab Ks 19.1 (IgG2a) (Progen, Heidelberg) reacts with cytokeratin component 19 [10].

Table 1. Immunocytochemical analysis of bone marrow aspirates in control patients without malignant disease

	Detection of cells with epithelial markers (%)
Mab directed against cell surface antigens	
$HMFG_1$ ($HMFG_1$)	32/75 (42.7)
$HMFG_2$ ($HMFG_2$)	8/75 (10.7)
EMA (E 29)	20/75 (26.7)
17-1A (M77/79)	0/20 (0.0)
Mab directed against cytokeratin components	
pan (Lu5)	0/20 (0.0)
8 (CK8)	0/20 (0.0)
18 (CK2)	0/75 (0.0)
19 (KS 19.1)	0/20 (0.0)

The antibodies were used at optimal concentrations ranging from 4–10 μg/ml. Appropriate dilutions of mouse myeloma proteins, UPC 10 (IgG2a) (Sigma) and MPC11 (IgG1) (Sigma) served as isotype controls.

The antibody reaction was developed with alkaline phosphatase using a polyvalent rabbit antiserum against mouse Ig and preformed complexes of alkaline phosphatase and monoclonal alkaline phosphatase antibodies (APAAP technique). Endogenous alkaline phosphatase was inhibited by preincubation with levamisole as described [11]. As with this procedure a background reaction was virtually absent, counterstaining could be omitted. Positive cells were clearly distinguishable by a strong colour reaction from unstained cells. Thus, a bone marrow smear was scored positive when one or more stained cells were detected. Each bone marrow aspirate was routinely screened by two independent observers in a blinded fashion.

For histology undecalcified bone marrow biopsies (average length 18 mm; diameter 3 mm) were embedded in synthetic resin and cut in 3 μm sections [12].

RESULTS

Reactivity of monoclonal antibodies with bone marrow aspirates from non-tumour-bearing patients

In a comparative approach we used antibodies directed either against cell surface antigens or against intracytoplasmic cytokeratin components on the same bone marrow preparations. In a first step bone marrow of non-cancer patients was analysed. As demonstrated in Table 1, freshly isolated haematopoietic cells of patients without known epithelial malignancies were neither stained by a broad-spectrum cytokeratin antibody (Lu5) nor by antibodies against individual cytokeratin components 8 (CK 8), 18 (CK2) or 19 (Ks19.1). When Mab directed against cell surface antigens were assessed, only M77 and M79, recognising epitopes of the 17-1A antigen, were non-reactive with bone marrow cells. In contrast, the antibodies HMFG1, HMFG2 and EMA, reacting with a mucin-like macromolecule, stained a small

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Table 2. Reactivity of mab against cytokeratin components in bone marrow aspirates of patients with breast, colorectal and gastric cancer

	Positive reactions (%)
Cell surface antigens	
17-1A (Mab M77/79)	6/50 (12.0)
Cytokeratin components	
pan (Mab Lu5)	10/50 (20.0)
8 (Mab CK8)	10/50 (20.0)
18 (Mab CK2)	11/50 (22.0)
19 (Mab K_S 19.1)	11/50 (22.0)

number of haematopoietic cells (0.5–10%) in 10.7–42.7% of 75 non-cancer patients. Some of the reactive cells showed a distinct plasma cell-like morphology.

Reactivity of monoclonal antibodies with bone marrow aspirates from patients with epithelial malignancies

In 50 patients with breast, colorectal or gastric cancer, bone marrow aspirates were analysed by the broad-spectrum cytokeratin antibody Lu5 and by each of three antibodies against cytokeratin components 8 (Mab CK8), 18 (Mab CK2) and 19 (Mab Ks19.1). Epithelial cells could be detected in 20–22% of the examinated patients (Table 2). In 10 of the 11 positive patients the bone marrow preparations reacted with all four anticytokeratin monoclonal antibodies, while 1 of the 11 patients exhibited a positive reaction with antibodies CK2 and Ks 19.1 only. Using a combination of 17-1A specific antibodies (M77/M79) we could identify epithelial cells only in 6 of the analysed 50 patients (12%).

As to the specificity and sensitivity of the procedure, cytokeratin associated determinants seem to be superior to membrane markers. For our further analysis of patients with adenocarcinoma we preferred the Mab CK2, which recognises cytokeratin component 18 selectively expressed in simple epithelia and adenocarcinoma cells derived thereof.

Detection of micrometastatic tumour cells in bone marrow aspirates of gastric cancer patients

Bone marrow samples, aspirated from the iliac crest of 97 patients with gastric carcinoma at the time of primary surgery, were examined with the Mab CK2 as outlined under methods. 34 of 97 (35.1%) marrow samples of patients with all tumour stages were positive for epithelial cells. These CK2-positive cells were found mostly as individual cells in a frequency of 10^{-4} to 10^{-5} nucleated bone marrow cells. The incidence of positive findings varied among patients and depended apparently on the extent of the tumour as indicated by involvement of regional lymph-nodes and presence of clinical manifest metastasis (Table 3). Cytokeratin-positive cells were found with a higher incidence (38.9%) in bone marrow aspirates of patients with histological evidence of locoregional lymph-node involvement than in those without positive lymph-nodes (21.6%). According to the histological classification of Laurén [13] gastric carcinoma can be divided into an intestinal and a diffuse type. When stratifying the patients according to this histological classification, patients with the diffuse type of gastric cancer exhibited a distinctly increased incidence of CK2-positive cells in their bone marrow (Table 4).

Table 3. Epithelial tumour cells in bone marrow of patients with gastric cancer (n = 97)

	Cytokeratin (CK2) positive cells
No distant metastases (M_0)	22/73 (30.1%)
$T_{1-4}N_0$	8/37 (21.6%)
$T_{1-4}N_{1-2}$	14/36 (38.9%)
With distant metastases (M_1)	12/24 (50.0%)
Total	34/97 (35.1%)

Table 4. Histological classification of gastric cancer (Laurén) and frequency of tumour cells in bone marrow (n = 93)

	Cytokeratin (CK2) positive cells
Intestinal type	17/57 (29.8%)
Diffuse type	11.25 (44.0%)
Mixed type	4/11 (36.4%)

To further investigate the sensitivity of the immunocytological assay, we simultaneously examined bone marrow specimens from 36 gastric cancer patients both by conventional histology and immunocytochemistry. The patients were divided according to presence (M_1) or absence (M_0) of clinically manifest distant metastasis (Table 5). In 1 of the 10 M_1 patients, metastasis was detected by conventional histology of a marrow biopsy not employing immunocytochemical staining. In the aspirates of this and 5 other patients, whose biopsies were negative by conventional histology, cytokeratin-positive cells were found. Of 26 patients in stage M_0 all were negative by histology, whereas 9 of them had cytokeratin-positive cells in their marrow.

To assess the biologic and clinical meaning of these findings, follow-up analyses of the patients were carried out. For such a clinical follow-up study 38 of 97 patients could be evaluated who fulfilled the following criteria: complete resection of the primary tumour and absence of clinically manifest distant metastases at the time of primary surgery (M_0). During a mean observation time of 25.4 months, 23 of 38 patients experienced a relapse in liver (16×), at locoregional sites (13×), in lung (2×) or in bone (2×). When these patients were classified according to their bone marrow findings, 12 relapses occurred in 13 CK2-positive patients, whereas 11 relapses were observed in 25 CK2-negative patients (Fig. 1), thus yielding a highly significant ($P = 0.002$) difference between the two groups according to the Kaplan–Meier analysis. Table 6 shows several prognostic features (lymph-node involvement, histolgical classification,

Table 5. Comparison of bone marrow histology and immunocytology in gastric cancer patients (n = 36)

	M_0	M_1
Histology positive	0/26 (0%)	1/10 (10%)
Immunocytology (MabCK2) positive	9/26 (34.6%)	6/10 (60%)

M_0 = distant metastases absent and M_1 = distant metastases present.

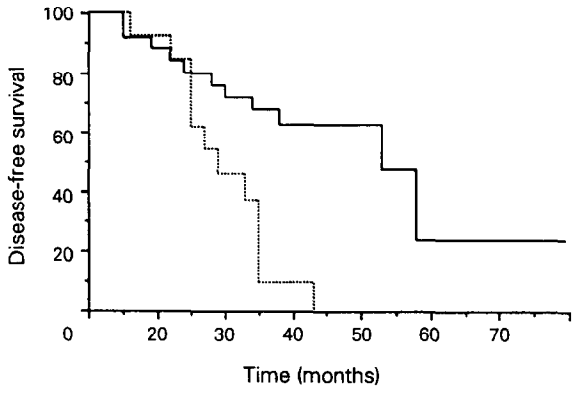

Fig. 1. Cytokeratin-positive cells in bone marrow and disease-free survival (locoregional and distant relapses) in 38 gastric cancer patients (P = 0.002, Kaplan-Meier analysis). Mean observation time: 25.4 months. ——— = cytokeratin-positive cells absent; · · · · = cytokeratin-positive cells present.

detection of CK2-positive cells) in relation to relapse. In spite of the small number of patients the division of N_0 patients into a low and a high-risk group may be clinically important.

DISCUSSION

One of the most pressing questions of modern oncology is how to detect and to eliminate occult micrometastatic tumour cells. Methods are needed which allow the detection of such cells at a time when they have not yet developed into gross and incurable macrometastases.

Applying an immunocytochemical technique, Redding *et al.* detected tumour cells in bone marrow smears in 28.2% of breast cancer patients without distant metastases at the time of primary surgery [14]. In their study, bone marrow aspirated from eight separate sites was stained with a polyclonal antiserum directed against the epithelial membrane antigen (EMA) which is related to or identical with the human milk fat globule antigen (HMFG). As demonstrated here, EMA and HMFG antibodies crossreact with epitopes on haematopoietic cells, particularly on lymphoid and plasma cells. In the meantime, the antigen defined as EMA or HMFG has been cloned and identified as a high molecular weight mucin-like glycoprotein which is profusely secreted into the medium [15]. Thus, also passive uptake of EMA or HMFG by other cells may explain the observed crossreactivity. Since cell size is a poor criterion for malignant cells it is difficult to distinguish between malignant and benign cells on morphological criteria. These results are in line with previously published studies [16–19].

Table 6. Frequency of relapses in 38 patients with gastric cancer according to stage/ histology and presence of cytokeratin (CK2) positive cells

	Relapse (%)
Stage	
No $CK2^-$	2/14 (14.3)
No $CK2^+$	4/5 (80.0)
N_{1-2} $CK2^-$	9/11 (81.8)
N_{1-2} $CK2^+$	8/8 (100.0)
Histology	
Intestinal $CK2^-$	7/16 (43.7)
Intestinal $CK2^+$	6/7 (85.7)
Diffuse $CK2^-$	3/6 (50.0)
Diffuse $CK2^+$	4/4 (100.0)
Mixed $CK2^-$	1/3 (33.3)
Mixed $CK2^+$	2/2 (100.0)

$CK2^-$ = cytokeratin-positive cells absent.
$CK2^+$ = cytokeratin-positive cells present.

Recently, an aberrant expression of cytokeratin components 8 and 18 was also seen in some non-epithelial tissues and cell-lines [20]. In our experience, however, freshly isolated haematopoietic cells could be stained neither by broad spectrum cytokeratin antibodies nor by antibodies directed against the individual cytokeratin components 8, 18 and 19. The restriction of the CK staining to epithelial cells was demonstrated by our group by double staining with anti-CD45 (leucocyte common antigen) antibodies which did not react with CK^+ cells [21]. Subsequent studies by other investigators, also examining bone marrow smears of cancer patients and patients without malignant disease, confirmed the specificity of cytokeratin staining [19, 22–24]. Being an essential constituent of the cytoskeleton of both normal and malignant epithelial cells, intracytoplasmic cytokeratin is a reliable marker for the epithelial origin of cells [5]. The original pattern of intermediate filaments, as expressed in the non-transformed state, is conserved with high fidelity in primary tumours as well as in metastatic nodules, while other epithelial cell surface antigens are only expressed by variable proportions of tumour cells.

As previously demonstrated for patients with mammary and colorectal cancer, the APAAP procedure, in combination with the cytokeratin specific monoclonal antibody CK2, improves greatly the detection of tumour cells in bone marrow as compared to conventional cytology and histology [21, 25, 26]. Since this antibody defines the cytokeratinic protein no 18, selectively expressed in cells of simple epithelia and adenocarcinoma derived thereof, it could also be used for the identification of gastric tumour cells in bone marrow. In gastric cancer, a tumour with an unfavourable prognosis due to an early systemic metastasis, the skeleton is, after the liver, the most frequent site of distant relapse [27]. Thus, it was not surprising to detect cytokeratin-positive cells in bone marrow aspirates of gastric cancer patients at such a high frequency. The presented data clearly show a correlation to established risk factors, particularly to the most important, the involvement of locoregional lymph-nodes. It is also interesting that the histological classification according to Laurén can apparently be correlated to marrow involvement. The two forms of gastric carcinoma appear to represent different biological entities in so far as the diffuse or poorly differentiated scirrhous gastric carcinoma recurs more frequently in bone than the intestinal type [27]. In accordance with this clinical observation we found cytokeratin-positive cells more frequently in bone marrow of patients with the diffuse form of gastric carcinoma.

As to the sensitivity of the assay, about one tumour cell in 10^4–10^5 bone marrow cells could be reproducibly detected. In the present study, the excellent sensitivity was verified by comparing the immunocytological technique with conventional bone marrow histology. As the distribution of metastasising cells in the bone marrow can not be assumed to be homogenous, the sensitivity of the detection system may be increased by analysing more bone marrow cells or multiple marrow aspirates as demonstrated in breast cancer patients (refs 14, 21 and I.F. *et al.*).

From a clinical point of view, an important aspect of the findings is their possible prognostic significance. Similar to the findings in colorectal cancer patients [26], gastric cancer patients, presenting with disseminated cytokeratin-positive cells at the time of primary surgery, experienced significantly more relapses than those patients without such cells. In spite of the noted crossreactivity of the EMA antibody with autochthonous bone marrow cells Mansi *et al.* [28] also reported a higher incidence of relapses in breast cancer patients exhibiting EMA^+ cells in their marrow. In our patients with gastric cancer the major site of relapse was the liver. It appears that in most patients cytokeratin-positive cells in bone marrow will not develop into manifest metastases within the time course of their disease, which is often terminated by complications arising from liver metastasis. The presence of such cells in bone marrow, however, may serve as valuable indicator of the intrinsic metastatic activity of an individual tumour.

Although the number of patients is too small to perform a multivariate analysis a combination of established risk factors, like locoregional lymph-node involvement and histological classification of the primary tumour, with immunocytochemical bone marrow screening appears to allow a distinct division of patients into different prognostic subgroups and may point to an independent prognostic significance of bone marrow screening.

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